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Award Number: DAMD17-99-1-9281

TITLE: Estrogen-Mediated Breast Carcinogenesis: The Role of Sulfation Pharmacogenetics

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CONTRACTING ORGANIZATION: Mayo Foundation
Rochester, Minnesota 55905

REPORT DATE: May 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2000	Annual (1 May 99 - 30 Apr 00)

4. TITLE AND SUBTITLE
Estrogen-Mediated Breast Carcinogenesis: The Role of Sulfation Pharmacogenetics

5. FUNDING NUMBERS
DAMD17-99-1-9281

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

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13. ABSTRACT ***(Maximum 200 Words)***

The carcinogenic effects of estrogen as a result of receptor -mediated mechanisms are well established. However, a growing body of evidence suggests that estrogens may also be direct genotoxins. Specifically, the catecholestrogens (CEs), 2- hydroxyestrone (2-OHE1), 2-hydroxyestradiol (2-OHE2), 4- hydroxyestrone (4-OHE1) and 4-hydroxyestradiol (4-OHE2) are estrogen metabolites that can be metabolically activated to semiquinones and quinones and form stable and depurinating DNA adducts. The depurinating DNA adducts are formed from 4-OHE1 and 4-OHE2 and they result in mutations that lead to genotoxicity and therefore breast carcinogenesis. Prevention of the genotoxic effects of these estrogen metabolites can be achieved in part through the sulfate-conjugation of the CEs, catalyzed by Sulfotransferase (SULT) enzymes. Many of the human cytosolic SULTs are genetically polymorphic, thus, inherited differences in the activities of these enzymes may contribute to the pathophysiology of breast cancer. As such, we determined the activity of 13 recombinant human SULTs with both 4-OHE1 and 4-OHE2, and also with the other CEs, 2-OHE1 and 2-OHE2 and the parent estrogens, estrone (E1) and 17-β estradiol (E2). All but one of the SULTs studied catalyzed these reactions to varying degrees. SULT 1E1 had the highest affinity for the CEs, with apparent Km values of 0.31 and 0.18 uM for 4-OHE1 and 4-OHE2 respectively. These results suggest that any individual variation in the sulfate conjugation of CEs catalyzed by SULTs may represent a risk factor for breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Pharmacogenetics, Sulfation, Sulfotransferases, Catecholestrogens	18
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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TABLE OF CONTENTS

INTRODUCTION

The carcinogenic effects of estrogen as a result of receptor -mediated mechanisms are well established. However, a growing body of evidence suggests that estrogens may also be direct genotoxins. Specifically, the catecholestrogens (CEs), 2- hydroxyestrone (2-OHE1), 2-hydroxyestradiol (2-OHE2), 4- hydroxyestrone (4-OHE1) and 4-hydroxyestradiol (4-OHE2) are estrogen metabolites that can be metabolically activated to semiquinones and quinones and form stable and depurinating DNA adducts. The depurinating DNA adducts are formed from 4-OHE1 and 4-OHE2 and they result in mutations that lead to genotoxicity and therefore breast carcinogenesis. Prevention of the genotoxic effects of these estrogen metabolites can be achieved in part through the sulfate-conjugation of the CEs, catalyzed by sulfotransferase (SULT) enzymes. Many of the SULTs are genetically polymorphic, thus, inherited differences in the activities of these enzymes would imply variations in enzyme activities towards the inactivation of the CEs. This would therefore lead to variations in the formation of the genotoxins that result in carcinogens-- ultimately contributing to the risk of breast cancer.

BODY

Task 1 was to determine which of the known human SULT isoforms and allozymes are capable of catalyzing the sulfate –conjugation of the catecholestrogens 2-OHE1, 2-OHE2, 4-OHE1 and 4-OHE2. Recombinant SULT proteins developed in our laboratory was used for the Substrate Kinetic studies. A latter addition to this Task was the inclusion of the parent estrogens, Estrone (E1) and Estradiol (E2), whose metabolites are the CEs. 1-6 months.

This Task has been completed. The modified method of Foldes and Meek for assaying SULTs was used for the substrate kinetic studies.

Figures 1 and 2 and Tables 1, 2, 3 and 4 in the Appendix are the results obtained from this study. **THESE RESULTS ARE IN A MANUSCRIPT BEING PREPARED FOR PUBLICATION.**

An **ABSTRACT** pertaining to this work can also be found in the appendix. This Abstract was chosen for an oral presentation at the American Society for Clinical Pharmacology and Therapeutics (ASCPT) annual meeting held in California, in March 2000, and it received the 2000 Presidential Trainee Award for the Society.

Task 2 was to use immunohistochemical techniques to localize the SULT isoform(s), which show relatively high affinities for the CEs, in normal and neoplastic breast tissues. 6-12 months.

Immunohistochemical studies were performed initially using SULT1E1 antiserum and 20 samples of paraffin-embedded breast cancer tissue sections, and 10 samples of frozen sections of normal breast tissue. Results showed that there was non-specific background staining, and therefore it was difficult to classify the staining as positive or negative. Because of the ambiguity of the results, a decision was made to purify the antiserum.

This polyclonal antiserum had not been originally purified, therefore a purification procedure using Protein A Sepharose CL-4B from Pharmacia Biotech was used to isolate and purify the IgG, which was then lyophilized.
To verify that the purified antibody was indeed that for SULT1E1, a Western blot was performed with recombinant SULT1E1. After ECL detection, results showed a single distinct band around 35KD, the known size of SULT1E1. This purified and lyophilized SULT1E1 antibody will be reconstituted to a higher concentration and used to repeat the immunohistochemical assays. If required, further affinity purification with recombinant SULT1E1 protein will be performed.

Task 3 was to test the hypothesis that there are functionally significant genetic polymorphisms within the specific SULT gene(s) identified and to develop allele-specific restriction digestion assays for these sequences to enable rapid genotyping. 7-24 months.
The Task 3 project as described in the Statement of work has not been initiated, but some work has been done to initiate the Task. Task 3 is the follow up of Task 2, where the SULT with highest affinity for the catecholestrogen, (SULT1E1) is to be used for immunohistochemical studies for possible localization. As explained in Task 2, there was such ambiguity in the results that I have recently purified the SULT1E1 antibody, which will be used to repeat the immunohistochemistry studies.
As a step towards accomplishing Task 3 and also as a step towards identifying the polymorphisms that may be present in SULT1E1, I have started the resequencing of this SULT. Resequencing is defined as seeking the common variant alleles of a gene(s), which are functionally important. Resequencing is used to describe the process of PCR amplification of many DNA samples (about 100) for all the exons and sections of introns immediately 5' and 3' to the exons in the gene(s) of interest, followed by sequencing analyses and calculation for allele-frequencies. At this time, instead of isolating DNA from normal breast tissues samples, we have available, DNA (M90PDR) from the Coriell Cell Repositories – i.e. the DNA Polymorphism Discovery Resource. This DNA resource comprises DNA from different ethnic backgrounds (25% each, African Americans, Native American, Hispanics and Caucasians). These samples have been striped of all identifiers. A total of 90 DNA samples is provided in M90PDR. This is the DNA source that has been recommended for use in the recently funded UO1grant for the Pharmacogenetic Research Network and Knowledge Base, of which Dr. Weinshilboum, a co-investigator on this DOD Grant and in whose laboratory I reside, is a recipient.
SULT 1E1 has eight (8) exons and these have been amplified using intron-based primers located approximately 100 to 150bp 5' and 3' of each of the exons. The exons were amplified with M13-tagged sequences for subsequent dye-primer sequencing--- a sequencing method that can identify unequivocally the heterozygous nature of amplicons. In addition, presence or absence of any insertion or deletion events can be identified. Analysis of the amplified sequences is being performed with a Computer program called Polyphred. This program helps to accurately identify heterozygous sites in sequences produced with fluorescence-based chemistries. The program compares sequence traces and searches for homozygotes and heterozygotes by the detection of a significant drop in fluorescence peak height at a variant site when sequence traces obtained from both homozygote and heterozygote individuals are compared.

Information obtained from all 90 samples is rapid so that in this particular case "relative allele frequencies" for the single nucleotide polymorphisms (SNPs) identified can be calculated. In addition, those SNPs, which will change encoded amino acids, can be tested for activity and functional significance. Thus knowing the genotype and the activity it elicits will lead to genotype – phenotype correlations. Overall, the results from this resequencing project will complement Specific Aim's 3 and 4 of this grant, so that after identifying the SNPs and determining which have functional importance, allele-specific digestion assays can be developed rapidly for use in these two aims-3 and 4.

KEY RESEARCH ACCOMPLISHMENTS

Task 1 was completed.
An oral presentation of the results was given at the 101st Annual meeting of the American Society for Clinical Pharmacology and Therapeutics (ASCPT), in March 2000, at Los Angeles, CA.
The Abstract received the 2000 Presidential Trainee Award for ASCPT.

REPORTABLE OUTCOMES

A manuscript entitled CATECHOL ESTROGEN METABOLISM: POSSIBLE ROLE FOR SULFATION AND CARCINOGENESIS is in preparation.

CONCLUSIONS

As a step towards identifying whether sulfate –conjugation catalyzed by SULTs may represent an independent risk factor for the development of breast cancer, we have determined that the catecholestrogens are substrates for most of the SULTs.
We also determined that SULT 1E1 had the highest affinity for 4-OHE2, the CE responsible for forming the genotoxins that form carcinogens.

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APPENDICES

Appendix I :

SULFATE CONJUGATION AND ESTROGEN-MEDIATED CARCINOGENESIS.
A.A. Adjei*, T.C.Wood*, P.C. Roche* and R.M. Weinshilboum.
Mayo Fdn., Rochester, MN.

The carcinogenic effects of estrogens as a result of receptor-mediated mechanisms are well established, but a growing body of evidence indicates that estrogens may also be direct genotoxins. Specifically, catecholestrogens (CEs) such as 4-hydroxyestrone (4-OHE1) and 4-hydroxyestradiol (4-OHE2) are estrogen metabolites that can be metabolically activated to quinones which can form depurinating DNA adducts. Prevention of the genotoxic effects of these estrogen metabolites can be achieved, in part, by the sulfate conjugation of CEs catalyzed by sulfotransferase (SULT) enzymes. Many human SULTs are genetically polymorphic, so inherited differences in the activities of these enzymes might contribute to the pathophysiology of breast cancer. Therefore, we have determined the activity of 13 recombinant human SULTs with both 4-OHE1 and 4-OHE2 as substrates. All but one of the enzymes studied could catalyze these reactions to varying degrees, but SULT1E1 had the highest affinity for the CEs, with apparent Km values of 0.31 μM for 4-OHE1 and 0.18 μM for 4-OHE2. We have also localized SULT1E1 to benign human breast tissue by immunohistochemical methods. These results indicate that individual variation in the sulfate conjugation of CEs catalyzed by SULTs may represent a risk factor for breast and other cancers.

Supported with Grant DAMD17-99-1-9281

Appendix II : See attached Figures and Tables on pages 12-17.

N.B.
ALL FIGURES AND TABLES IN APPENDIX II ARE PROPRIETARY DATA.

Figure 1

Estrogen-Induced Carcinogenesis
Possible Role for Sulfation

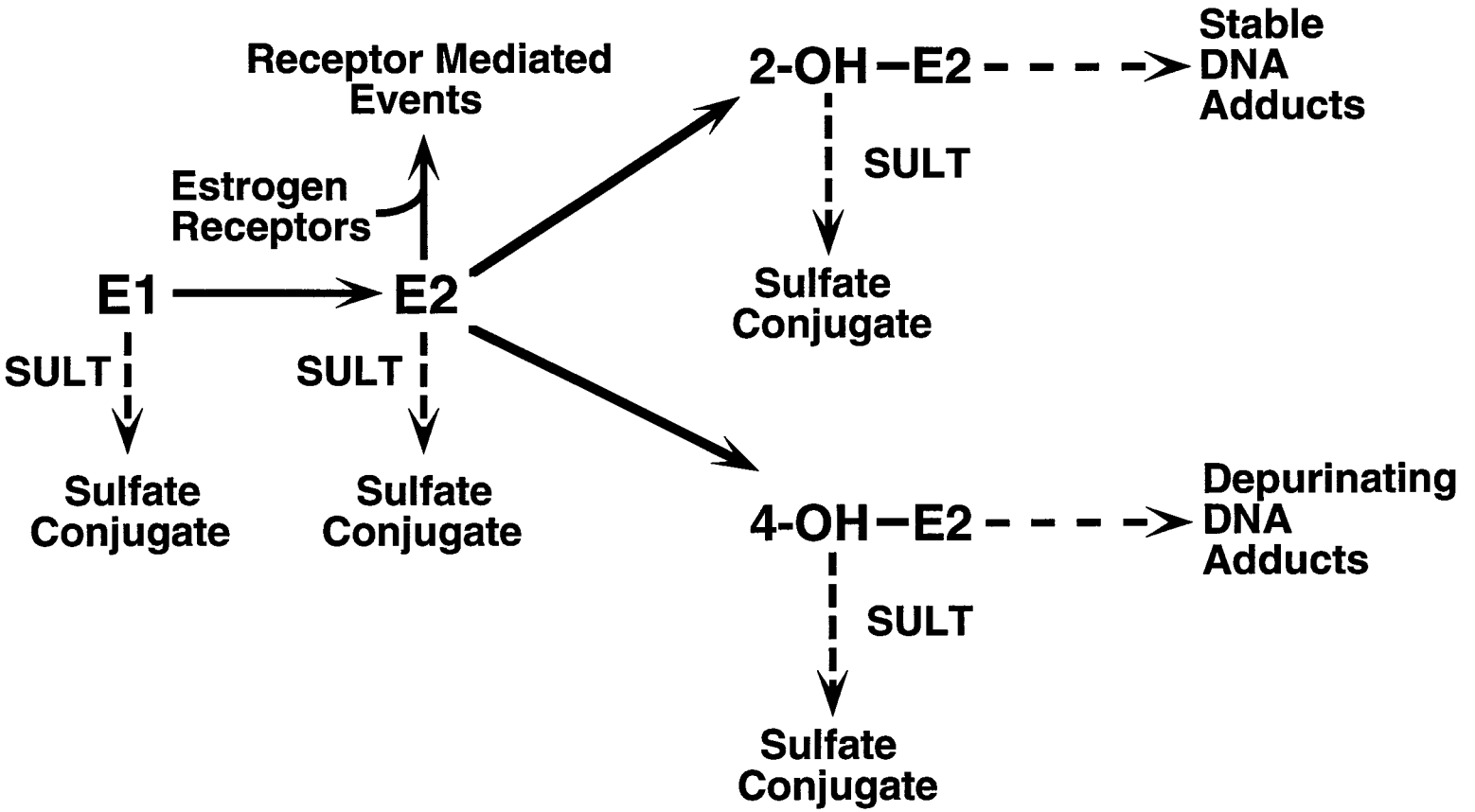

Figure 2

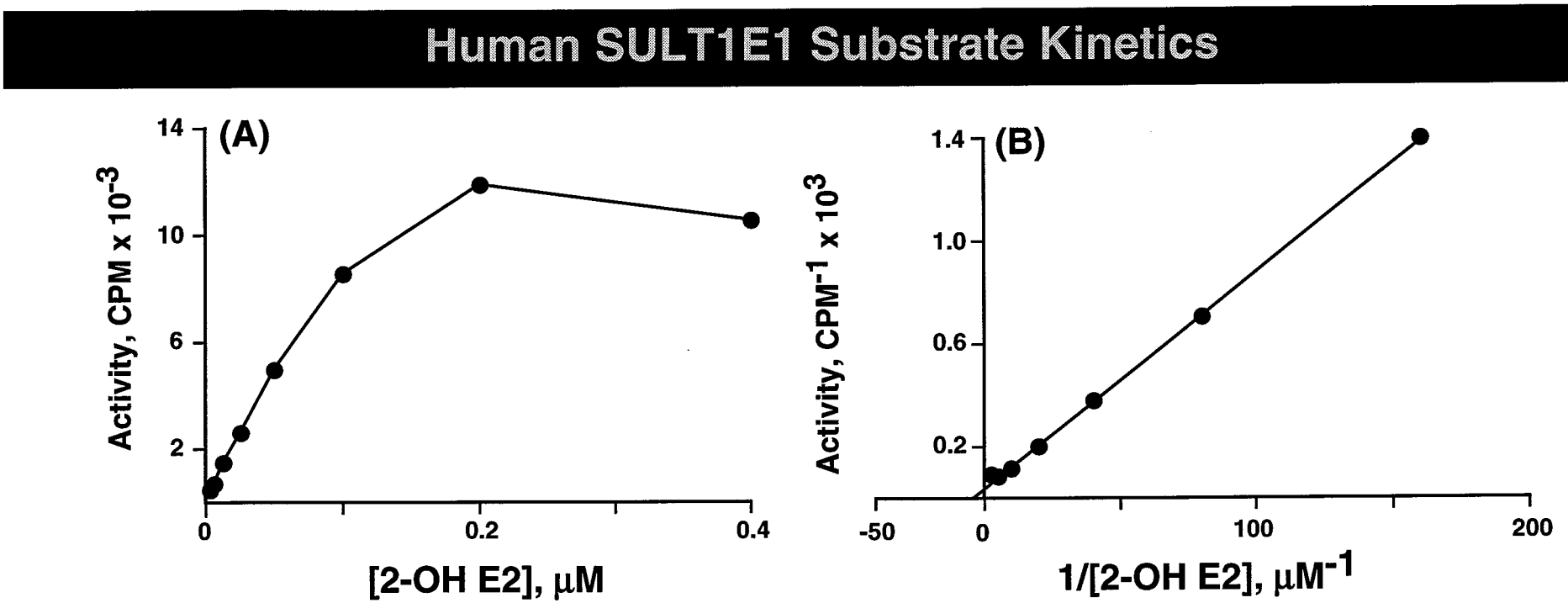
Human SULT1E1 Substrate Kinetics
(A)
Activity, CPM x 10-3
14
10
6
2
0
0.2
0.4
[2-OH E2], µM
(B)
Activity, CPM-1 x 103
1.4
1.0
0.6
0.2
-50
0
100
200
1/[2-OH E2], µM-1

Table 1

Estrogen-Induced Carcinogenesis
K_m Values for 2-OH E1 (E2)

SULT Isoform	Apparent K_m Values (μM ± S.E.)	
	2-OH E1	2-OH E2
1A1*1	5.3 ± 1.3	2.5 ± 0.2
1A1*2	3.6 ± 2.8	11.6 ± 0.5
1A1*3	3.6 ± 0.4	17.3 ± 2.0
1A2*1	19.6 ± 2.4	10.7 ± 1.6
1A2*2	31.5 ± 3.2	484.5 ± 75.1
1A2*3	8.9 ± 1.2	40.8 ± 2.6
1A3	188.4 ± 4.0	81.8 ± 6.9
1C1	ND	ND
1E1	0.27 ± 0.08	0.22 ± 0.03
2A1	12.7 ± 1.3	2.4 ± 0.1
2B1a	6.7 ± 1.5	3.3 ± 0.4
2B1b	3.3 ± 0.5	2.5 ± 0.1

ND = no detectable activity

Table 2

Estrogen-Induced Carcinogenesis
K_m Values for 4-OH E1 (E2)

SULT Isoform	Apparent K_m Values (μM ± S.E.)	
	4-OH E1	4-OH E2
1A1*1	53.8 ± 2.1	44.0 ± 5.2
1A1*2	53.3 ± 9.3	23.4 ± 3.7
1A1*3	40.4 ± 4.4	6.3 ± 0.1
1A2*1	17.1 ± 2.0	27.9 ± 0.7
1A2*2	65.1 ± 9.4	42.4 ± 2.7
1A2*3	5.1 ± 0.8	7.6 ± 2.0
1A3	32.4 ± 3.7	475.8 ± 93.8
1C1	ND	ND
1E1	0.31 ± 0.13	0.18 ± 0.06
2A1	20.4 ± 1.0	41.5 ± 19.5
2B1a	20.7 ± 1.8	45.2 ± 13.0
2B1b	13.6 ± 0.4	17.5 ± 1.0

ND = no detectable activity

Table 3

Estrogen-Induced Carcinogenesis
Km Values for E1 and E2

SULT Isoform	Apparent Km Values μM ± S.E.	
	E1 (ESTRONE)	E2 (ESTRADIOL)
1A1*1	ND	31.3 ± 6.2
1A1*2	ND	84.6 ± 24.3
1A1*3	ND	21.1 ± 2.9
1A2*1	ND	28.6 ± 3.8
1A2*2	ND	145.4 ± 21.3
1A2*3	ND	18.3 ± 2.2
1A3	ND	ND
1C1	ND	ND
1E1	0.11 ± 0.1	0.097 ± 0.06
2A1	11.2 ± 0.9	12.4 ± 0.9
2B1a	ND	84.3 ± 7.6
2B1b	ND	60.9 ± 10.0

ND - Little or no detectable activity .

Table 4

SULT1E1 Substrate Kinetics

Substrate	K_m (μM)	V_{max} (Units/mg protein)	V_{max}/K_m
E2	0.097	6.88	70.9
E1	0.11	4.13	37.5
4-OH E2	0.18	6.04	33.6
2-OH E2	0.22	4.77	21.7
4-OH E1	0.31	2.35	7.6
2-OH E1	0.27	1.61	6.0

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